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HER2 immunohistochemistry in endometrial and ovarian clear cell carcinoma: discordance between antibodies and with *in-situ* hybridisation

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HER2 immunohistochemistry in endometrial and ovarian clear cell carcinoma: discordance between antibodies and with *in-situ* hybridisation

Aims: Treatment with anti-HER2 therapy could be beneficial for patients with HER2-positive endometrial and ovarian clear cell carcinoma (CCC). We studied HER2 overexpression by immunohistochemistry (IHC) using three different antibodies, including concordance with amplification by *in-situ* hybridisation (ISH).

Methods and results: IHC and ISH were performed on tissue microarrays of 101 tumours: 58 endometrial pure CCC, 19 endometrial mixed carcinomas with a CCC component and 24 ovarian pure CCC. IHC was performed using SP3, 4B5 and HercepTest antibodies, and was scored by two independent observers. ISH was performed using dual-colour silver ISH. Using IHC, agreement was poor between SP3/4B5 (61.4%), poor between SP3/HercepTest (68.3%) and reasonable between 4B5/HercepTest (75.2%). Interobserver agreement was substantial to almost perfect for all antibodies (SP3: linear weighted $\kappa = 0.89$, 4B5:

$\kappa = 0.90$, HercepTest: $\kappa = 0.76$). HER2-positivity by ISH was 17.8% (endometrial pure CCC: 24.1%, endometrial mixed: 0%, ovarian pure CCC: 16.7%). IHC/ISH concordance was poor, with a high false-negative rate of all three IHC antibodies: sensitivity (38.9–50.0%) and positive predictive value (PPV) (37.5–58.3%) were poor; specificity (81.9–94.0%) and negative predictive value (NPV) (87.1–88.3%) were reasonable. When excluding 2+ cases, sensitivity declined (26.7–43.8%) but PPV (80.0–87.5%) and specificity (98.6–98.7%) improved.

Conclusions: In ovarian and endometrial CCC, there is considerable difference in HER2 overexpression by different IHC antibodies and marked discordance with ISH. As such, no single antibody can be considered conclusive for determining HER2 status in CCC. Based on these results, the lack of predictive value of different HER2 testing methods, as used in other studies, could be explained.

Keywords: endometrial cancer, human epidermal growth factor 2 (HER2), immunohistochemistry (IHC), *in-situ* hybridisation (ISH), ovarian cancer

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Introduction

Endometrial and ovarian cancer are common malignancies in women, responsible for 4% (endometrial) and 5% (ovarian) of cancer-related female deaths.¹ Most endometrial and ovarian cancers are epithelial

tumours, mainly endometrioid and serous carcinoma.^{2–4} The third subtype is clear cell carcinoma (CCC), accounting for up to 2–5% of endometrial and 5–25% of ovarian carcinomas.^{4–8} CCC is a high-grade adenocarcinoma, associated with aggressive clinical behaviour and poor prognosis.^{8–10} Additionally, a ‘mixed’ endometrial carcinoma subtype exists, containing at least 5% of multiple subtypes.¹¹ In endometrial mixed carcinoma with a serous component, tumour behaviour correlates with the highest grade (serous) component. A CCC component in mixed carcinoma might therefore be clinically relevant, but few data are available on these tumours.^{11,12} The mixed carcinoma category for ovarian carcinoma was abandoned in the most recent World Health Organization (WHO) classification (2014).¹¹

Treatment options for endometrial and ovarian CCC are limited. Response to traditional treatment with surgery, radiotherapy or chemotherapy is poorer than other high-grade carcinomas.^{5–7} Molecular alterations in CCC are being identified, and strategies with targeted therapies are still in development.^{9,13} The current concept of precision cancer medicine strives for a patient tailored approach. Therefore, it is crucial to define individual tumour characteristics. A possible target for therapy is the human epidermal growth factor receptor 2 (HER2/ErbB2), well known from its clinical significance in HER2-positive breast- and gastroesophageal cancer.^{14–22} In endometrial and ovarian cancer, HER2-positivity varies considerably: 17–80% in endometrial and 8–66% in ovarian carcinoma.^{23–25} Specific data on CCC are scarce, with 14–67% HER2-positivity reported in small patient cohorts (often $n < 10$).^{25–37} Results of anti-HER2 therapy in endometrial and ovarian carcinoma to date are inconsistent.^{31,36–46} No clinical data of anti-HER2 therapy in CCC are available. However, the definition of HER2-positivity between studies varies widely.

HER2 status can be determined by quantifying cell membrane overexpression with immunohistochemistry (IHC) or by assessing gene amplification with *in-situ* hybridisation (ISH). ISH is considered the ‘gold standard’ in breast- and gastroesophageal cancer, with high predictive value of IHC.^{22,47} However, HER2 testing is not standardised in endometrial and ovarian carcinoma. Additionally, IHC overexpression by different HER2 antibodies can vary.^{26,48–50} Although concordance between HER2 antibodies in breast- and gastroesophageal cancer is high,^{22,47,51} this has not been established for endometrial and ovarian cancer. Studies on endometrial and ovarian

CCC to date have applied different criteria, using either IHC or ISH and often not both.^{25–37} Studies with IHC used various HER2 antibodies and none have compared different antibodies.

The aim of this study was to compare HER2 overexpression by different IHC antibodies and their concordance with HER2 amplification by ISH in a large cohort of patients with endometrial and ovarian CCC.

Materials and Methods

PATIENTS

Formalin-fixed paraffin-embedded tumour tissue available for tissue microarray (TMA) was obtained from curettages, biopsies or resection specimens of patients with endometrial CCC (pure or mixed with a CCC component) treated in the University Medical Center Groningen (UMCG, the Netherlands) in 1984–2016 or in the Isala Hospital Zwolle (the Netherlands) in 2006–2016, and of patients with ovarian CCC treated in the UMCG in 2000–2012. All cases were reviewed by two gynaecopathologists to confirm the diagnosis of pure CCC or mixed carcinoma with a CCC component (endometrial cases: H.H. and J.B.; ovarian cases: H.H. and E.D.; only cases with a concordant diagnosis between both pathologists were included). This yielded 103 patients: 24 ovarian pure CCC, 60 endometrial pure CCC and 19 endometrial mixed carcinomas, 18 of which were mixed endometrioid/CCC [CCC component: mean = 36% ($\pm 23.5\%$ standard deviation; range = 5–80%)] and one mixed serous/CCC (CCC component: 20%). All ovarian samples contained pure CCC. During the course of the study two cases were excluded due to unsuccessful ISH, resulting in a study population of 101 patients (Figure 1, patient characteristics in Table 1).

Patient material was handled following the ‘Code of conduct for health research’ of the Dutch Federation of Biomedical Scientific Societies.⁵² Data were filed in a separate anonymous database. Therefore, no additional permission from our Ethics Committee was required.

TMA CONSTRUCTION AND HER2 CONTROLS

TMAs were constructed using a manual microarrayer (Beecher Instruments, Silver Spring, MD, USA); two TMAs with 0.6 mm endometrial tumour cores, one TMA with 1 mm ovarian tumour cores. Of every patient, three to six tumour cores were obtained. This included three selective cores of both components in

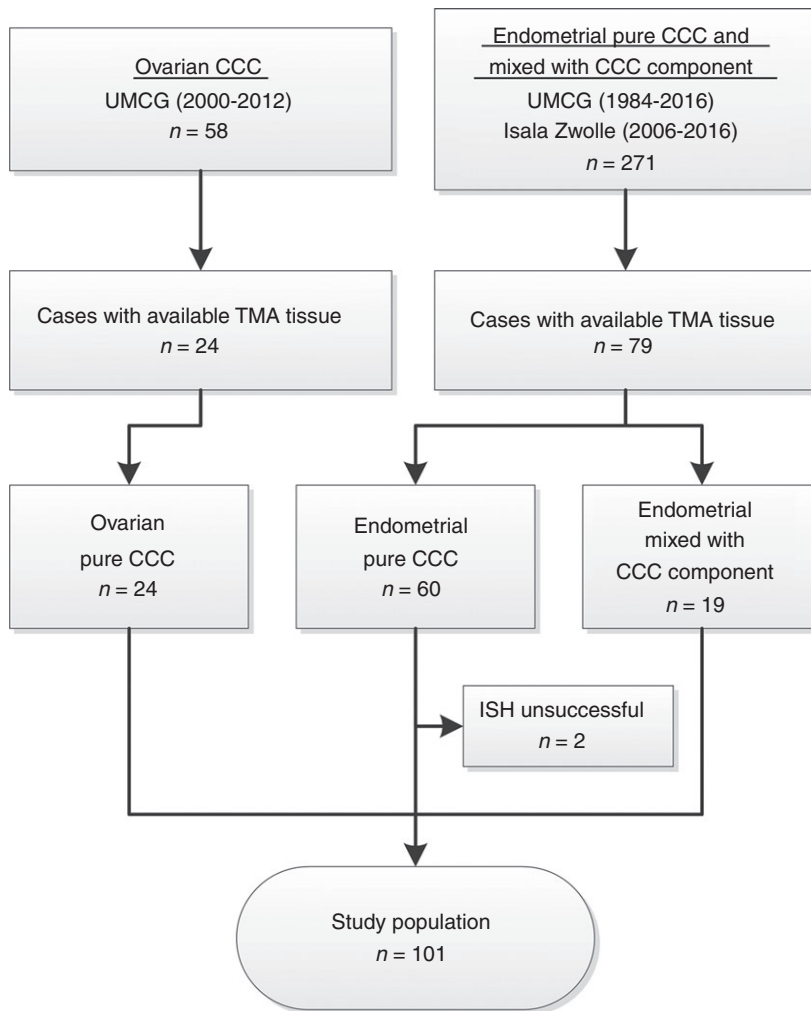


Figure 1. Flow-chart of the study population formation.

mixed carcinomas. Tissues from various other organs were included as controls. Standardised HER2 controls were included in all IHC and ISH tests (Breast Dynamic Range Analyte Control; HistoCyte Laboratories, Newcastle upon Tyne, UK). Uniform staining of these controls was assured on all slides. 3- μ m sections were cut for IHC and ISH.

IMMUNOHISTOCHEMISTRY

IHC for 4B5 (PATHWAY anti-HER2/*neu* (4B5) rabbit monoclonal antibody; Ventana Medical Systems, Illkirch, France) and SP3 (rabbit monoclonal antibody; NeoMarkers, Fremont, CA, USA) were performed on the Ventana BenchMark Ultra. 4B5 was prediluted, SP3 diluted 1:40. Antigen retrieval time was 64 min (95°C, cell conditioning 1, pH 9; Ventana); incubation time with the primary antibody was 32 min. Visualisation was achieved with the

ultraView diaminobenzidine detection kit (Ventana), including antigen amplification (Ventana Amplification Kit). Counterstaining was performed with Mayer's haematoxylin (Klinipath, Breda, the Netherlands).

IHC for HercepTest (rabbit polyclonal antibody A0485; Dako, Carpinteria, CA, USA) was performed in the Autostainer Link 48 (Dako) using the HercepTest Kit SK001 (Dako). This includes antigen retrieval (40 min, 97°C) in PT Link (Dako), incubation with the primary antibody (30 min) and incubation with the visualisation complex (30 min). Counterstaining was performed with Mayer's haematoxylin (SK308; Dako).

All three antibodies are used in daily clinical practice on breast- and gastroesophageal cancer, following the manufacturer's protocols with standardised staining kits (as described above). SP3 and 4B5 are used in the UMCG; HercepTest is used in the

Table 1. Patient characteristics

	Total population (<i>n</i> = 101)	Endometrial pure CCC (<i>n</i> = 58)	Endometrial mixed w/CCC component (<i>n</i> = 19)	Ovarian pure CCC (<i>n</i> = 24)
Age at diagnosis (years)				
Mean (\pm SD)	66 (\pm 12)	70 (\pm 10)	63 (\pm 16)	59 (\pm 9)
Range	39–92	44–89	39–92	45–83
FIGO stage (<i>n</i> , %)				
I	44 (44.9)	30 (54.5)	7 (36.8)	7 (29.2)
II	10 (10.2)	5 (9.1)	2 (10.5)	3 (12.5)
III	36 (36.7)	13 (23.6)	9 (47.4)	14 (58.3)
IV	8 (8.2)	7 (12.7)	1 (5.3)	0 (0)
Unknown	3	3	0	0

CCC, Clear cell carcinoma; FIGO, International Federation of Gynaecology and Obstetrics; SD, Standard deviation.

Maasstad Hospital (Rotterdam, the Netherlands). All antibodies were validated internally, including comparison with ISH results. SP3 and HercepTest have been subjected to external proficiency testing with NordiQC in 2017, with 'good' and 'optimal' results. Additionally, adequate antibody performance during the study was ensured on the standardised HER2 controls.

IN-SITU HYBRIDISATION

Dual-colour silver ISH was performed with the INFORM HER2 Dual ISH DNA probe cocktail (Ventana) on the Ventana BenchMark Ultra. Pretreatment was with cell conditioning 2 (Ventana; 36 min, 86°C, three cycles) and enzyme digestion with ISH protease 3 (12 min), followed by incubation with HER2 (dinitrophenol-labelled) and chromosome 17 (CEP17, digoxigenin-labelled) probes for 4 min. Probes were denatured (20 min, 80°C) and hybridised (6 h, 80°C), followed by appropriate stringency washes (three times, 8 min, 74°C). The HER2 probe (black dots) was visualised by incubation with anti-dinitrophenol antibody (20 min) and horseradish peroxidase-conjugated antibody (32 min) followed by silver reactions (8 min). The CEP17 probe (red dots) was visualised by incubation with anti-digoxigenin antibody (20 min) and alkaline phosphatase-conjugated antibody (24 min) followed by red ISH Naphthol reaction (8 min). Slides were counterstained with Ventana hematoxylin II and bluing reagent. Adequate ISH performance was ensured on the standardised HER2 controls.

IHC AND ISH EVALUATION

IHC stains of 4B5, SP3 and HercepTest were scored independently by an experienced pathologist (B.V.) and a senior resident (T.K.). HER2 expression was graded using the standard semi-quantitative scale;⁵³ 0: no staining or membrane staining in \leq 10% of tumour cells; 1+: faint/barely perceptible partial membrane staining in $>$ 10%; 2+: weak-to-moderate complete membrane staining in $>$ 10%; and 3+: strong complete membrane staining in $>$ 10%. The highest IHC score by either observer was used as the score for the case.

ISH was evaluated according to current breast cancer guidelines⁴⁷ by calculating the HER2/CEP17 ratio and the average HER2 copy number in 20 tumour cells: negative: ratio $<$ 2.0, copy number $<$ 4.0; positive: ratio \geq 2.0 or ratio $<$ 2.0, copy number \geq 6.0; and equivocal: ratio $<$ 2.0, copy number \geq 4.0/ $<$ 6.0. In equivocal cases, 20 additional tumour cells were counted.

For both IHC and ISH, the highest score in one of the TMA cores was considered representative for the case (in accordance with the 10% cut-off).⁴⁷

Heterogeneity was defined as either a different ISH result, or IHC score difference of $>$ 1 point with at least one antibody by at least one observer.

P 53

Additional p53 IHC was performed on all endometrial carcinomas, using the anti-p53 monoclonal mouse antibody BP53-11 (Ventana) on the Ventana

Benchmark Ultra, following the manufacturer's protocol. p53 staining was differentiated in physiological wild-type expression and mutational overexpression. Overexpression was defined as diffuse strong staining in tumour cell nuclei.

STATISTICS

For agreement between observers, linear weighted kappa (κ) statistics were performed in R for Windows version 3.3.2 (R Foundation, Vienna, Austria), using the 'irr' package. κ values were interpreted as <0.2, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; and 0.81–1.00, almost perfect agreement.⁵⁴ For IHC/ISH concordance, IHC results were compared to ISH as the 'gold standard'. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), including 95% confidence intervals, were calculated in two analyses. In the first analysis IHC 2+ cases were considered positive, because 2+ is commonly considered positive in literature on HER2 in endometrial and ovarian carcinoma. In the second analysis IHC 2+ cases were excluded, as in breast- and gastroesophageal cancer 2+ is 'equivocal' and not predictive of ISH amplification.^{22,47} p53 overexpression rates in HER2-positive and -negative endometrial carcinomas were compared using Fisher's exact test (two-sided, $P < 0.05$ considered significant) in IBM SPSS Statistics for Windows version 23.0.0.3 (SPSS, Inc., Chicago, IL, USA).

Results

IMMUNOHISTOCHEMISTRY

IHC results for the three different antibodies are displayed in Table 2. Agreement between SP3 and 4B5 was 61.4%; between SP3 and HercepTest was 68.3%; and between 4B5 and HercepTest was 75.2%. Examples of concordant cases are shown in Figure 2, discordant cases in Figure 3. The SP3 antibody showed more staining and resulted in higher scores than 4B5 and HercepTest. 4B5 showed more cases without any staining (score 0) than SP3 and HercepTest. These discrepancies occurred in both observers, with high interobserver agreement: 89.1% (SP3, $\kappa = 0.89$; 'almost perfect'), 89.0% (4B5, $\kappa = 0.90$; 'almost perfect') and 81.2% (HercepTest, $\kappa = 0.76$; 'substantial') (Table S1). The discordance between antibodies occurred in both endometrial and ovarian pure CCC, with both observers (Table S2).

Table 2. Comparison of IHC scores using different HER2 antibodies

		4B5				Total
		0	1	2	3	
SP3	0	44	1	1	0	46
	1	22	9	0	0	31
	2	1	11	4	0	16
	3	0	1	2	5	8
	Total	67	22	7	5	101
Agreement: 61.4%						
		HT				Total
		0	1	2	3	
SP3	0	40	6	0	0	46
	1	12	17	2	0	31
	2	1	8	7	0	16
	3	0	1	2	5	8
	Total	53	32	11	5	101
Agreement: 68.3%						
		HT				Total
		0	1	2	3	
4B5	0	51	15	1	0	67
	1	2	15	5	0	22
	2	0	2	5	0	7
	3	0	0	0	5	5
	Total	53	32	11	5	101
Agreement: 75.2%						

IHC, Immunohistochemistry; HER2, Human epidermal growth factor 2; SP3, SP3 antibody; 4B5, 4B5 antibody; HT, HercepTest antibody.

HER2 PREVALENCE

HER2-positivity by IHC and ISH is displayed in Table 3. By ISH, HER2-positivity was 17.8% in the total study population ($n = 101$); 24.1% in endometrial pure CCC ($n = 58$), 0% in endometrial mixed carcinoma with a CCC component ($n = 19$) and 16.7% in ovarian pure CCC ($n = 24$). The ISH amplification ratios of HER2-positive cases are shown in Table S3. All TMA cores of all components of the

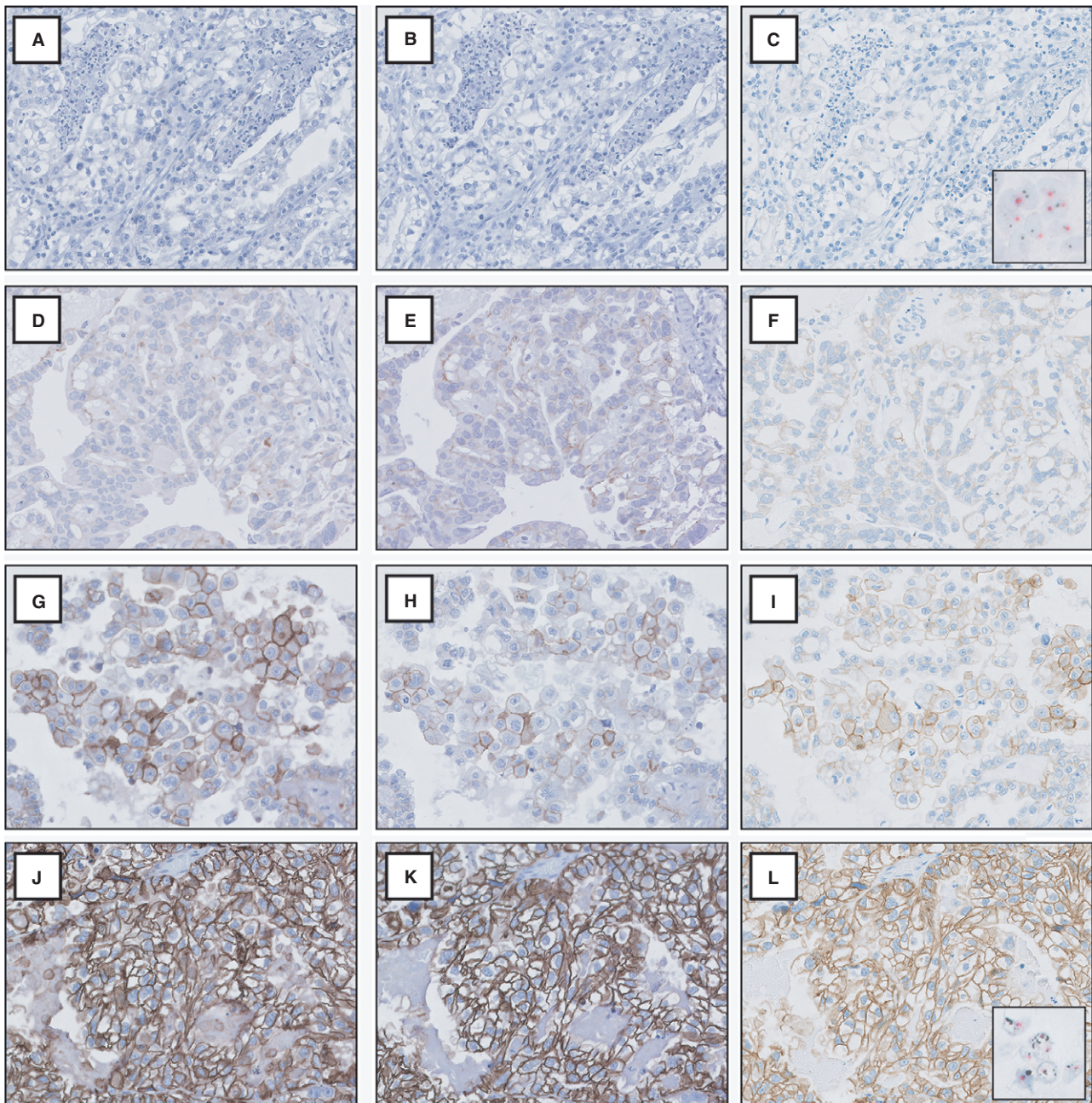


Figure 2. Human epidermal growth factor 2 (HER2) immunohistochemistry (IHC) with concordant staining between different antibodies: SP3 (left row), 4B5 (middle row) and HercepTest (right row) with score 0 (A–C), score 1+ (D–F), score 2+ (G–I) and score 3+ (J–L). Insets: examples of negative *in-situ* hybridisation (ISH) (diploid: inset of C) and positive *in-situ* hybridisation (ISH) (amplified: inset of L).

endometrial mixed carcinomas were HER2-negative by ISH and by IHC (0/1+) with all three antibodies.

IHC/ISH CONCORDANCE

IHC/ISH concordance is shown in Table S4. Sensitivity, specificity, PPV and NPV of all IHC antibodies

with ISH as a reference are shown in Table 4. Including 2+ scores, there was a high false-negative rate of IHC by all three antibodies, resulting in poor sensitivity (38.9–50.0%) and PPV (37.5–58.3%) with reasonable specificity (81.9–94.0%) and NPV (87.1–88.3%). When excluding 2+ scores, many ISH-negative 2+ cases were excluded, and consequently,

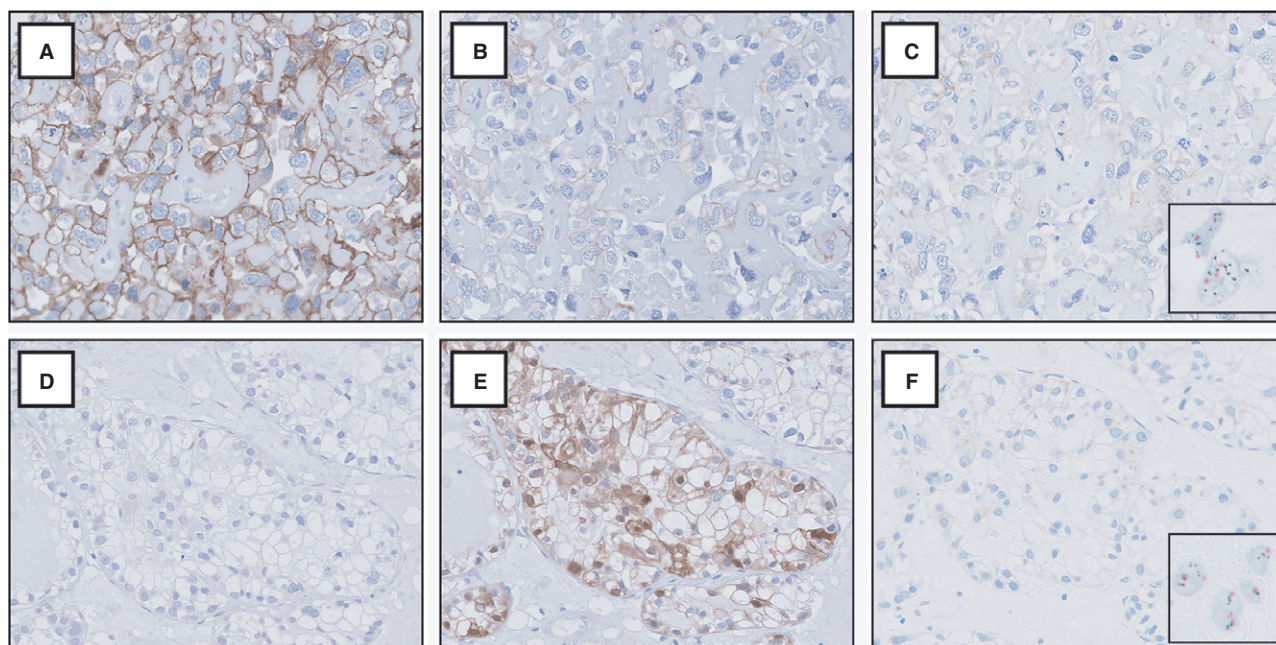


Figure 3. Discordant human epidermal growth factor 2 (HER2) immunohistochemistry (IHC) between different antibodies. Example 1 showed 2+/3+ staining with SP3 (A) but was 1+ with both 4B5 (B) and HercepTest (C). Example 2 was completely negative (score 0) with SP3 (D) but showed 2+ staining with 4B5 (E) and faint 1+ staining with HercepTest (F). *In-situ* hybridisation (ISH) was positive in both cases (amplified: insets of C and F).

Table 3. HER2-positivity by different criteria in the total study population and in subgroups

HER2-positive criterion	Total population (<i>n</i> = 101)	Endometrial pure CCC (<i>n</i> = 58)	Endometrial mixed with a CCC component (<i>n</i> = 19)	Ovarian pure CCC (<i>n</i> = 24)
ISH amplified	18 (17.8%)	14 (24.1%)	0 (0%)	4 (16.7%)
IHC 2+/3+, SP3	24 (23.8%)	21 (36.2%)	0 (0%)	3 (12.5%)
IHC 2+/3+, 4B5	12 (11.9%)	9 (15.5%)	0 (0%)	3 (12.5%)
IHC 2+/3+, HT	16 (15.8%)	13 (22.4%)	0 (0%)	3 (12.5%)

HER2, Human epidermal growth factor 2; CCC, Clear cell carcinoma; ISH, *in-situ* hybridisation; IHC, Immunohistochemistry; SP3, SP3 antibody; 4B5, 4B5 antibody; HT, HercepTest antibody.

sensitivity declined (26.7–43.8%) but PPV (80.0–87.5%) and specificity improved (98.6–98.7%), with identical NPV (87.1–88.3%).

HETEROGENEITY

Heterogeneity between TMA cores of the same tumour occurred in only nine cases (8.9%), either as a different ISH result (*n* = 3) or IHC score difference of >1 point with at least one antibody by at least one observer (*n* = 6).

P 5 3

Results of p53 IHC, performed on all endometrial carcinomas, are displayed in Table 5. In endometrial mixed carcinomas, p53 staining was identical in both components of all tumours. Interestingly, p53 overexpression was much more frequent in HER2-positive endometrial pure CCC than in HER2-negative endometrial pure CCC (11 of 13 = 84.6% versus 15 of 40 = 37.5%; *P* = 0.002) and HER2-negative endometrial mixed carcinoma (11 of 13 = 84.6% versus three of 19 = 15.8%; *P* < 0.001). There was

Table 4. Sensitivity, specificity and predictive values (in %) for IHC using different antibodies with ISH as a reference

	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	Total (<i>n</i>)	2+ (<i>n</i> , %)
Including cases with IHC 2+ score						
SP3	50.0 (26.8–73.2)	81.9 (71.6–89.2)	37.5 (19.6–59.2)	88.3 (78.5–94.2)	101	16 (15.8%)
4B5	38.9 (18.3–63.9)	94.0 (85.9–97.8)	58.3 (28.6–83.5)	87.6 (78.6–93.4)	101	7 (6.9%)
HT	38.9 (18.3–63.9)	89.2 (79.9–94.5)	43.8 (20.8–69.4)	87.1 (77.6–93.1)	101	11 (10.9%)
	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	Total (<i>n</i>)	
Excluding cases with IHC 2+ score						
SP3	43.8 (20.8–69.4)	98.6 (91.1–99.9)	87.5 (46.7–99.3)	88.3 (78.5–94.2)	85	
4B5	26.7 (8.9–55.2)	98.7 (92.2–99.9)	80.0 (29.9–98.9)	87.6 (78.6–93.4)	94	
HT	26.7 (8.9–55.2)	98.7 (91.8–99.9)	80.0 (29.9–98.9)	87.1 (77.6–93.1)	90	

IHC, immunohistochemistry; ISH, *in-situ* hybridisation; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; SP3, SP3 antibody; 4B5, 4B5 antibody; HT, HercepTest antibody.

Table 5. p53 overexpression in endometrial carcinoma cases

	Pure CCC, HER2-positive	Pure CCC, HER2-negative	Mixed carcinoma,* HER2-negative
p53 wild-type	2 (15.4%)	25 (62.5%)	16 (84.2%)
p53 overexpressed	11 (84.6%)	15 (37.5%)	3 (15.8%)
Missing	1	4	–
Total	14	44	19

CCC, Clear cell carcinoma; HER2, Human epidermal growth factor 2.

*All endometrial mixed carcinoma cases were HER2 negative.

no significant difference in p53 expression between HER2-negative endometrial pure CCC and HER2-negative endometrial mixed carcinoma (15 of 40 = 37.5% versus three of 19 = 15.8%; $P = 0.132$).

Discussion

Targeted treatment of the HER2 receptor might be beneficial for patients with HER2-positive CCC. In a large cohort of patients with endometrial or ovarian CCC, we compared HER2 overexpression by IHC using different antibodies and established their concordance with ISH amplification. To the best of our knowledge, this is the first study to compare different HER2 antibodies in these tumours. We found

discordance between three antibodies. For all antibodies the predictive value of HER2 amplification by ISH was poor.

Data on HER2-positivity in endometrial and ovarian CCC are scarce. Studies on endometrial carcinoma included few CCC (usually $n < 10$), among which HER2-positivity varied between 16 and 67% (IHC and ISH).^{25–30,37} In a larger cohort of 58 endometrial pure CCC cases, we found 24.1% HER2-positivity by ISH and 15.5–36.2% by IHC (2+/3+), which is in line with these studies. Larger studies are available for ovarian CCC ($n = 5–92$), showing 14–46% HER2-positivity rates by IHC or ISH.^{31–36} We found 16.7% HER2-positivity by ISH and 12.5% by IHC (2+/3+) in 24 ovarian pure CCC cases, which is lower than or comparable to these studies. To the best of our knowledge, no studies exist on HER2-positivity in endometrial mixed carcinoma with a CCC component. Interestingly, we found that in this subgroup of our study ($n = 19$), all tumours were HER2-negative in all components by both ISH and IHC. The carcinogenic pathways leading to pure CCC and mixed carcinoma therefore seem to differ, and it seems unlikely that patients with endometrial mixed carcinoma with a CCC component will benefit from anti-HER2 therapy.

The difference of HER2-positivity rates reported in the literature could be due to different methods and criteria used to establish HER2 status. ISH amplification is considered the 'gold standard' in breast- and gastroesophageal cancer,^{55–57} with high predictive value of IHC overexpression^{57,58} and high

concordance between antibodies.^{22,47,51} IHC score 0/1+ is considered negative; 2+ equivocal (subsequent ISH should follow); and 3+ positive. In contrast, HER2 testing is not standardised in endometrial and ovarian carcinoma, and concordance between HER2 antibodies has not been established. Most studies, including endometrial and ovarian CCC, applied IHC without ISH, often with 2+/3+ scores considered positive, and none have compared IHC antibodies (most use HercepTest). Although no data on endometrial CCC are specifically available, IHC/ISH discordance is known to occur in endometrial carcinoma in general, with higher overexpression than amplification rates.^{24–26} On ovarian CCC, only one study implemented IHC and ISH on all cases, reporting 14% HER2-positivity (six of 50 cases) with perfect IHC/ISH concordance (2+/3+ classified positive with HercepTest).³⁴ In our study, IHC/ISH concordance was poor in both endometrial and ovarian CCC, even when we excluded IHC 2+ scores (in which discordance with ISH can be expected due to the equivocal nature of 2+). Although this occurred with all three antibodies, there were also considerable differences between these antibodies. Possible explanations for different antibody performances are individual antibody sensitivity and specificity, but also technical differences in staining methods (such as dilution, epitope retrieval method and incubation times).^{26,48–50} Additionally, pre-analytical factors such as tissue size, fixative type, fixation time and temperature during fixation and processing could influence antibody sensitivity.⁵⁹ We included samples from only two laboratories, in which tissue processing has been consistent for years. To circumvent technical artefacts, our study included standardised HER2 controls in which uniform antibody behaviour was assured.

The inconsistency of IHC and ISH could also explain the inconsistent results of studies on anti-HER2 therapy in endometrial and ovarian carcinoma to date. Anti-HER2 therapy is an established part of breast- and gastroesophageal cancer treatment.^{14–22} Trastuzumab was shown to reduce ovarian CCC cell lines *in vitro*,³⁶ but no clinical data of anti-HER2 therapy in endometrial or ovarian CCC are available. In other endometrial carcinoma subtypes, clinical activity of trastuzumab has been described in several case reports.^{38–40} Several Phase II studies on endometrial carcinoma^{37,41} as well as ovarian carcinoma^{31,42–46} have shown a poor response to anti-HER2 therapy with trastuzumab, pertuzumab or lapatinib, but these studies did not include CCC or did not specify response per subtype. The reason for these poor results could be inconsistent HER2 testing among

these studies, which applied enzyme-linked immunosorbent assay (ELISA),⁴² reverse transcription–polymerase chain reaction (RT–PCR),⁴³ IHC without ISH^{31,38,39,41,45} or ISH without IHC.^{40,42} One study did not establish HER2 status at all.⁴⁴ Some studies included HER2-negative patients.^{41,42,45} The only clinical study which included CCCs using both IHC and ISH based HER2-positivity on IHC (2+/3+ with HercepTest), while almost half these patients (15 of 33; 45.5%) lacked ISH amplification.³⁷ This study included only three endometrial CCCs and did not specify results within this subgroup. Very recently, encouraging results with anti-HER2 therapy were achieved in a Phase II study on endometrial serous carcinoma, where the addition of trastuzumab to standard chemotherapy increased progression-free survival in patients with HER2-positive tumours.⁶⁰ In this study, HER2-positivity was defined as an IHC 3+ score, or IHC 2+ with ISH amplification. However, the IHC antibody was not specified, while the differences in antibody performance in endometrial CCC, as shown in our study, might also be a concern in endometrial serous carcinoma.

In addition to differences in HER2 testing, another mechanism which may explain poor response to anti-HER2 therapy is the loss of HER2 expression in metastases. This was shown in a recent study on endometrial cancer, which included 790 endometrial carcinomas, including 30 CCCs.⁶¹

Yet another possible explanation of the variable results of anti-HER2 therapy in endometrial and ovarian carcinoma is the mechanism leading to HER2 amplification. The amplification ratios of HER2-positive tumours in our study were generally lower than in breast cancer (25–29% of cases with HER2/CEP17 ratio >5.0, versus >50% in breast cancer). ISH amplification with relatively low amplification ratios may reflect aneuploidy or polysomy in carcinomas with high copy number abnormalities, rather than being the driver event with HER2 gene amplification seen in breast- and gastroesophageal cancer.

We found that p53 overexpression is more common in HER2-positive than in HER2-negative endometrial carcinomas. p53 mutations are associated with chromosomal instability, i.e. aneuploidy or polysomy, and with amplification instability, i.e. gene amplification.⁶² Co-existence of p53 and HER2 overexpression also occurs in breast cancer, in which HER2 overexpression is related to gene amplification.⁶³ In ovarian and endometrial carcinoma, p53 overexpression could be related to HER2 overexpression due to either aneuploidy or amplification; the underlying mechanism is unknown.

A potential limitation of our study is the use of TMAs, as TMA cores can miss relevant data and HER2 staining can be heterogeneous. Heterogeneity between TMA cores occurred in only nine (8.9%) of our study cases, but it is unknown whether this represents whole sections. However, studies have shown that taking at least three tumour cores, as was performed in our study, results in adequate representation of the whole section staining pattern and, as such, largely obviates heterogeneity issues.^{64,65} Moreover, the use of TMAs was not a limitation with regard to the comparison of IHC antibodies and IHC/ISH comparison, as identical cores were compared directly.

Although ISH is considered the 'gold standard' for HER2 status in breast- and gastroesophageal cancer, some patients with IHC-positive/ISH-negative tumours can benefit from anti-HER2 therapy and some patients with IHC-negative/ISH-positive tumours might not respond to anti-HER2 therapy.^{22,47,55–57} Anti-HER2 therapy has been successful in breast cancer patients with polysomy and normal HER2/CEP17 ratios.⁶⁶ A HER2-positive subgroup in patients with endometrial and ovarian CCC could benefit from anti-HER2 therapy, but it is unknown whether IHC or ISH would be a better predictor of clinical outcome.

In conclusion, we found considerable differences in HER2 overexpression by different IHC antibodies, as well as discordance with HER2 amplification by ISH, in a large cohort of patients with endometrial and ovarian CCC. Therefore, no single IHC antibody can be considered to be conclusive when determining HER2 status in these tumours. Based on these results the lack of predictive value of different HER2 testing methods, as used in other studies, can possibly be explained. As some patients may benefit from anti-HER2 therapy, future studies should include HER2 testing with different IHC antibodies as well as ISH, to evaluate effectively the best predictor of clinical response.

Conflict of Interests

None to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Comparison of HER2 IHC scores between both observers.

Table S2. Comparison of immunohistochemistry scores using different HER2 antibodies, by both observers and divided into tumor location and subtype.

Table S3. ISH amplification ratios in HER2-positive tumours, compared to breast cancer*.

Table S4. Concordance of immunohistochemistry using different HER2 antibodies with ISH, in the total study population and divided into tumor location and subtype.